Changes in CBF-BOLD Coupling Detected by MRI During and After Repeated Transient Hypercapnia in Rat

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ABSTRACT

The effect of hypercapnia on cerebral metabolic rate of oxygen consumption ($CMRO_2$) remains incompletely understood. This study examined the relationship between susceptibility (Blood Oxygenation Level Dependent, BOLD) and perfusion-weighted (Flow-sensitive Alternating Inversion Recovery, FAIR) MRI techniques both during induction of repeated transient hypercapnia (THC), and after return to normocapnia during whisker barrel functional activation. During induction of THC the FAIR signal became significantly elevated over control after 100 seconds of hypercapnia (p=0.039) with a trend of increasing significance to five minutes (p=0.000008). The FAIR signal in the activated cortex during subsequent normocapnia was significantly increased compared to pre-THC control after each successive period of THC. The FAIR signal increased by 81±63% after one exposure (p=0.021), by 163±55% after the second exposure (p=0.0002), and by 240±54% after the third exposure (p=0.000002). The BOLD signal did not increase significantly during, or after, exposure 1, 2, or 3. These data demonstrate increased uncoupling of perfusion-weighted from susceptibility imaging techniques, both in non-activated cortex during hypercapnia, and with activation after multiple exposures to THC. These results are consistent with saturation of BOLD contrast as well as with increases in $CMRO_2$ with stimulation after mulitiple exposures to THC.

Key words: functional MRI; hypercapnia; CBF; $CMRO_2$

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TABLE OF CONTENTS

INTRODUCTION

Background. Since Blood Oxygenation Level Dependent (BOLD) imaging was first described by Ogawa in 1990, it has become an important tool for the study of brain physiology using functional Magnetic Resonance Imaging (fMRI) (1, 2, 3, 4). fMRI employs advanced MRI techniques to investigate physiological changes in the brain that are related to neuronal activation, or a change in neuronal activity. BOLD contrast-based fMRI can be used to localize areas of increased neural activity in the brain caused by an external sensory stimulus because of the following key principles. Deoxyhemoglobin becomes strongly magnetized when placed in a magnetic field, and this magnetization in venous blood interferes with the acquisition of MRI signals from nearby tissue, resulting in a reduction of local signal intensity. The BOLD signal reflects a complex interplay of the intravascular magnetic susceptibility (which depends on the venous blood oxygenation of hemoglobin), blood flow, and blood volume (1, 2, 3). Things that decrease venous blood deoxyhemoglobin, such as increased blood flow and decreased blood volume, will result in increased BOLD signal. Increased deoxyhemoglobin, due to things like increased oxygen extraction and increased blood volume, will decrease the BOLD signal. Studies using near infrared spectrophotometry and intrinsic optical reflectance have shown that deoxyhemoglobin decreases after sensory stimulation onset in the adult mammalian brain (5, 6, 7). These studies are consistent with the increased BOLD signal observed in similar fMRI stimulation experiements (4, 8, 9, 10, 11).

More recently the use of perfusion-weighted imaging (P-WI), where blood flow is related to the signal intensity of each pixel, has increased as a complement to susceptibility imaging. The most commonly used arterial spin labeling methods are

related to the Flow-sensitive Alternating Inversion Recovery (FAIR) sequence, first described by Kim in 1995 (12). In FAIR imaging, two different types of images are acquired, one flow sensitive, the other flow insensitive. The perfusion-weighted image is obtained by subtraction of the flow insensitive image from the flow sensitive image. Refer to Figure 1 for a more detailed explanation of how P-WI are produced using the FAIR technique. A wide array of related research has also been conducted on other determinants of fMRI contrast, such as blood glucose level, and the proposed vasodilators nitric oxide and adenosine (8, 13, 14, 15).

Current Issues. Despite the widespread use of BOLD fMRI, several questions remain unanswered about the BOLD mechanism and the manner in which the signal depends on underlying physiological processes. One issue of particular interest and importance is the relationship between cerebral blood flow (CBF) and cerebral metabolic rate of oxygen consumption ($CMRO_2$) (9, 10, 11, 16, 17, 18, 19). Various models have been developed to account for this relationship. Buxton and Frank (18, 20) predicted that a very large change in CBF is necessary to support a small change in $CMRO_2$, while Hyder et al. (5, 21) predicted a much stronger correlation between CBF and $CMRO_2$. The theories of Buxton and Frank are based partially on the results of several positron emission tomography (PET) studies that demonstrated a much larger increase in CBF than $CMRO_2$ with sensory stimulation (22, 23). However, in many other PET studies changes in CBF and $CMRO_2$ were nearly proportional (24, 25, 26, 27). Hyder et al. based their predictions on a review of the literature that included PET, as well as other types of functional imaging modalities (5, 21).

An alternative to investigating this relationship with PET is to use MRI to collect

BOLD and P-WI while the subject undergoes graded visual stimulation (28, 29, 30). These studies were performed after "calibrating" the functional signal at what was presumed to be baseline $CMRO_2$ with transient hypercapnia (THC), on the assumption that the increase in CBF seen with hypercapnia would not be accompanied by a significant increase in $CMRO_2$. There is nearly universal agreement that hypercapnia significantly increases CBF (9, 10, 11, 12, 14, 15, 28, 30, 42). It therefore follows from the conflicting literature on the relationship of CBF and $CMRO_2$ that the assumption that $CMRO_2$ remains at baseline with THC is a controversial one. Many additional studies of the relationship between CBF and $CMRO_2$ have been performed with MRI, as well as other modalities, on many different organisms, with apparently conflicting results (14, 31, 32, 33, 34, 35, 36). For example, while a study by Kety and Schmidt did not demonstrate a significant increase in $CMRO_2$ during hypercapnia (31), a study by Hovarth et al. demonstrated a hypercapnia-induced increase in $CMRO_2$ of over 50% in rats, which was partially attenuated by L-NAME, a nitric oxide synthase inhibitor (14).

One finding that may further confound attempts to perform calibrated fMRI (but which may also potentially offer the possibility of increasing the sensitivity of functional P-WI) has been reported by Schmitz et al. (37) and Bock et al. (38). Bock et al. reported that in α–chloralose anesthetized rats, THC resulted in significantly increased P-WI signal in response to somatosensory stimulation under subsequent normocapnia 25 minutes later, when compared to the pre-THC flow response. No significant change was noted in the BOLD signal. If it is assumed that intravascular susceptibility and CBF, but not blood volume, are the dominant determinants of BOLD contrast in this system, which were the findings of Kennan et al. (39, 40) and Kida et al. (41), then these results suggest

hypercapnic up-regulation of $CMRO_2$ with somatosensory stimulation. This study was recently repeated in awake humans to assess whether THC would increase the functional P-WI signal in response to a visual search task (42). No significant increase in the P-WI signal was seen, so the findings from the earlier animal studies were attributed to an unknown mechanism of the anesthesia modulating the cholinergic neurotransmitter system.

Purpose & Outcome. In the present study the effects of THC on the P-WI and BOLD functional signal in α–chloralose anesthetized rats are more closely examined. The hypothesis states that if THC changes the functional-hemodynamic coupling of activated somatosensory cortex, then this change will be demonstrated with the fMRI methods employed in this study. By measuring the FAIR and BOLD signals during induction of THC in the cortex without stimulation, it is found that there is a significant increase in the FAIR signal after 100 seconds of THC, while the increase in the BOLD signal never becomes significant. The results of the present study from the stimulated cortex confirm those of Bock et al. (38), and furthermore, they demonstrate that there is early significant up-regulation of the flow response, but not the BOLD signal, after THC, as well as show that this flow response to somatosensory stimulation becomes more robust and increases significantly after multiple exposures to THC. This effect of THC is shown to be localized to the region of the cortex receiving stimulation by an analysis of images from the contralateral cortex.

The data demonstrate that THC changes the coupling of the functional-hemodynamic response after one exposure, and results in a progressively larger change with multiple exposures. One explanation that could account for these findings is that the

BOLD signal became saturated because of non-linear behavior of BOLD contrast at high oxygen saturations (48, 51). Considering the recent literature on the relationship of CBF and $CMRO_2$, the results of this study are also consistent with the theory which states that changes in CBF correlate with changes of similar magnitude in $CMRO_2$ (5, 21). Therefore, this study suggests that performing THC-calibrated fMRI in an attempt to quantify $CMRO_2$ may lead to spurious results.

METHODS

This study was conducted in accordance with institutional review board guidelines. All procedures, methods, and experiments described here were conducted by the author, Michael V. Dutka.

Animal Preparation. Four female Sprague Dawley rats weighing 220-300 g were initially anesthetized with ketamine/xylazine. They were then tracheotomized and artificially ventilated using 0.4-1.0% halothane in an oxygen/nitrogen mixture. A femoral vein was catheterized for intravenous infusion of α–chloralose during image acquisition. Intravenous infusion of α–chloralose was initiated during imaging protocol set-up, at least an hour before image acquisition, with a 60 mg/kg bolus. Maintenance doses of 20 mg/kg were administered every 0.5 hour, and as needed to maintain the mean arterial blood pressure under 110 mmHg. For muscle relaxation intra-peritoneal tubocurarine (0.5 mg/kg) was administered before image acquisition, then a maintenance dose (0.25 mg/kg) was given every hour. A femoral artery was catheterized for arterial blood pressure monitoring and collection of blood samples (0.7 cc each) for arterial blood gas measurements. A total of ten blood gas samples were collected during the entire time course of the study. To restrict movement, the head was gently fixed by an acrylic clamp and a bite bar inserted behind the front teeth. Temperature was monitored by a rectal probe and modulated by adjusting the flow to a hot water blanket and by blowing warm or cool air into the bore of the magnet.

To stimulate sensory input to neurons in the whisker barrel, the paradigm developed by Yang et al. (43) was utilized. Two needle electrodes were placed subdermally on the right cheek, one along the anterior border of the verbrissae, one along

the posterior border (5, 43). Whisker stimulation was applied using a gated Tektronix 2620 constant current stimulus isolator with 0.3 ms duration pulses with a frequency of 3 Hz and a current of 2 mA. Prior to neuromuscular blockade, application of the stimulus elicited fasciculations of the muscle underlying the verbrissae. To reduce electrical noise, the output of the stimulator was first filtered before the wires entered the bore of the magnet through a copper door.

Magnetic Resonance Imaging. Imaging was performed at 2.0 T on a Bruker Avance imaging system using a 72 mm birdcage coil for RF transmission, and an electrically and geometrically decoupled circular surface coil placed under the head for RF reception. Median sagittal scout images were obtained for transverse fMRI slice planning. Maximal whisker barrel activation was seen in the left sensory cortex, contralateral to the electrodes, when the anterior edge of the transverse slice was positioned 7.0-7.5 mm posterior to the most posterior part of the olfactory bulb. BOLD functional magnetic resonance imaging was performed using gradient echo Echo Planar Imaging, EPI (44) with an FOV of 50 mm, an acquisition matrix of 64X64, and a slice thickness of 2.0 mm, with TE = 50 ms, TR = 2.0 s, and the flip angle = 90°. For each BOLD image set, five dummy scans were performed, followed by 50 repetitions alternating between 10 images with stimulation and 10 images without stimulation. One scout image was also collected for use later in correcting for phase artifacts during reconstruction, resulting in a total BOLD acquisition time of 140 s. FAIR imaging was performed with the same geometry using gradient echo EPI, but with either a slice-selective (4 mm) or non-selective inversion recovery preparation. For inversion of spins an 8 ms duration 2000 Hz bandwidth adiabatic full passage pulse was used (45, 46). For

FAIR imaging, TI = 1.0 s, TE = 20 ms, and TR = 2.0 s. For each FAIR image set, five dummy scans were performed, followed by the acquisition of 60 selective, non-selective image pairs alternating between 10 image pairs with stimulation and 10 image pairs without stimulation, for a total of 120 repetitions. PW-I were calculated by subtracting the non-selective images from the slice-selective images on a pixel-by pixel basis (12). Two scout images were also collected, resulting in a total FAIR acquisition time of 280 s. In order to obtain simultaneous perfusion-weighted and BOLD information during induction of THC, BOLD effects during THC induction were measured from non-selective images from the FAIR sequence.

Figure 2 summarizes the experimental protocol, which was divided into 10 minute blocks, seven minutes were generally used for imaging (except during hypercapnia, where only FAIR images were acquired), with the remaining time equally divided before and after the imaging. This was done to minimize manipulations to the system during image acquisition, allowing time for blood gas sampling, anesthesia administration, and temperature modulation while minimizing neuronal fatigue. As a result the rat received a maximum 3 minutes net of stimulation per 10 minute block. Initially, 2 sets each of FAIR and BOLD images were collected while the rat was ventilated with a 40% O_2/ 60% N_2 mixture to establish a baseline before THC was induced. A blood sample was collected for blood gas analysis between the two image sets. Mild THC was induced by ventilating the rat with a 10% CO_2/ 90% O_2 mixture for 5 minutes. During induction of hypercapnia, a set of FAIR images was collected. After 5 minutes of THC another blood sample was collected and there was a 5 minute wash-out period during which the rat was ventilated with 40% O_2/ 60% N_2 at approximately 20% higher tidal volumes. This was

followed by four to six 10 minute blocks consisting of one set of BOLD images followed by one set of FAIR images as previously described. A blood gas sample was collected after the first image set, 13.5 minutes after cessation of hypercapnia. After a 10 minute pause, the above protocol was repeated two more times, beginning with the acquisition of one set of FAIR and BOLD images before the induction of mild hypercapnia, for a total of three runs following THC for each rat.

Analysis. Functional activation was analyzed using parametric maps based on the Student's *t* test. Maps were created for each set of images by subtracting the images without stimulation from the images with stimulation (47). The activated region of interest (ROI) was defined as the six most significantly activated contiguous pixels for each image set. Contiguous was defined as being connected by edges or by corners. Cortical signal intensity for the "stimulus off" images within a set was determined by manually selecting all pixels determined to be in the cortex of the hemisphere contralateral to the activated ROI. This selection was performed for one FAIR and one BOLD image set per rat and subsequently, the same pixel locations were used for all the other image sets from that particular rat. For each rat mean values from image sets were normalized to the corresponding values from the average of the pre-hypercapnia control images. For statistical analysis, all data were grouped into one of seven groups; Control, Hypercapnia 1,2 or 3, or post-hypercapnia Run 1, 2 or 3. The Student's paired *t*-test was used to test for statistical significance between groups. A p-value of <0.05 was defined as the level of statistical significance.

RESULTS

The physiologic parameters recorded before, during, and after the three periods of THC are shown in Table 1. No significant differences in arterial partial pressure of carbon dioxide (pCO_2) existed between the pre-THC runs and post-THC runs, or between the three periods of THC. After 5 minutes of ventilation with 10% CO_2 the mean increase in arterial pCO_2 was 30.6±1.2 mmHg (means ± SD), from 29.6±1.5 mmHg pre-THC to 60.2±0.7 mmHg immediately post-THC. THC caused a corresponding decrease in arterial blood pH of 0.21±0.02 units, from 7.27±0.01 pre-THC to 7.06±0.02 post-THC. Ventilation with the 10% CO_2/90% O_2 mixture also resulted in an increase in the arterial partial pressure of oxygen (pO_2) of 60.4±12.9 mmHg, from 122.2±5.3 mmHg to 182.6±8.8 mmHg. Body temperature and mean arterial blood pressure did not vary significantly throughout the entire experiment.

Figure 3 depicts a representative example of FAIR and BOLD data acquired from one animal during the entire time course of the experiment. Data were normalized to the pre-THC controls. Signal intensities from the unstimulated somatosensory cortex are included along with signal intensities elicited by stimulation. The FAIR signal from the unstimulated cortex increased 60-70% over control at the end of each five minute period of THC. The BOLD signal from the unstimulated cortex also increased with THC. Both the FAIR and BOLD signals from the unstimulated cortex returned to baseline by the second set of post-THC images. With stimulation the FAIR signal progressively increased after each period of THC. The BOLD signal with stimulation increased after the first exposure to THC, then leveled-off after the second and third exposures to THC.

Figure 4 shows the mean FAIR and BOLD signal intensity data from the

unstimulated cortical hemisphere grouped from all three runs during THC induction and normalized to the repective pre-THC signal intensities. At image 20 the FAIR signal became significantly elevated vs. pre-THC control and remained significantly elevated until the end of the run, with a trend toward greater significance with time as the FAIR signal increased during exposure to THC, with p values ranging from $p = 0.039$ for image 20 to $p = 0.000008$ for image 60. The BOLD signal reached a plateu at 100 seconds and did not significantly increase from pre-THC controls at any time throughout the induction period of THC.

Figure 5 shows FAIR and BOLD activation maps obtained during right whisker stimulation at normocapnia, before any CO_2 was administered (Control), and at normocapnia, 35 minutes after CO_2 was administered once (Run 1), twice (Run 2), and three times (Run 3). The maps are overlaid on the respective FAIR and BOLD EPI images aquired between periods of stimulation. In both FAIR and BOLD maps, the most significantly activated pixels were observed in the somatosensory cortex contralateral to the electrodes. The areas of greatest activation were in good spatial agreement between FAIR and BOLD activation maps. The level of activation in the FAIR maps significantly increased after each successive run of THC, as depicted by the marked increase in both the number of and strength of activation of significantly activated pixels. The level of activation in the BOLD maps showed a small, but not significant, increase after one exposure to THC, and did not appreciably increase after Run 2 or Run 3 post-THC. The time course data that Figure 5 represents is illustrated by Figure 6, which shows the corresponding time courses of relative changes in the FAIR and BOLD signals in the somatosensory cortex of one rat with stimulation.

Figures 7 summarizes the activation data from each of the four periods of normocapnia, which consisted of a pre-THC control, and runs following the first, second, and third exposures to THC. The data were averaged over all animals (except for post-THC Run 3 of one animal that had clearly become hemodynamically unstable) and grouped as pre-THC control, or post-THC Run 1, 2, or 3. The bars represent the averaged data normalized to the respective pre-THC control. In the control group stimulation produced a signal increase in the whisker barrel of 31.0±5.9% in the FAIR images and 1.84±0.40% in the BOLD images. After one exposure to THC the raw FAIR signal increased by 81±63% and the raw BOLD signal increased by 27±54% over control. After the second exposure to THC the raw FAIR signal increased by 163±55% and the raw BOLD signal increased by 41±67% over control. After the third exposure to THC the raw FAIR signal increased by 240±54% and the raw BOLD signal increased by 35±53% over control. All three FAIR runs significantly increased over control (p=0.021, p=0.0002, p=0.000002), while none of the BOLD runs were significantly increased (p=0.22, p=0.098, p=0.081). In addition the FAIR signal of post-THC Run 2 was significantly higher than the FAIR siganl of post-THC Run1 (p=0.011), and the FAIR signal of post-THC Run 3 was significantly higher than the FAIR signal of post-THC Run 2 (p=0.027). Cortical FAIR and BOLD signal intensity from the unstimulated hemisphere during 'off' images were also measured. There were no significant changes between Control, or Runs 1-3 for either FAIR or BOLD images.

Figure 8 shows only the first set of FAIR and BOLD data with stimulation collected from 6.5 to 13.5 minutes after the cessation of the first exposure to THC. The bars represent the averaged signal data normalized to the respective pre-THC control.

The FAIR signal intensity increased by 78±62% compared to control (p=0.0005). The BOLD signal intensity did not significantly increase from control (-5±18%; p=0.501).

DISCUSSION

These results show that there are significant increases in the FAIR signal, but not in the BOLD signal, both during hypercapnia without stimulation and after hypercapnia with stimulation. This can be interpreted in several ways depending on what are assumed to be the dominant determinants of the BOLD contrast. One explanation for why the BOLD signal failed to increase significantly during induction of THC, and also with subsequent stimulation, is that it became saturated. The literature on saturation, or the supposed inability of the BOLD signal to increase further because virtually all deoxyhemoglobin has been washed-out of the venules by high CBF, is equivocal. Bruhn et al. (48) showed that the BOLD signal was saturable when using acetazolamide to pharmacologically increase CBF in humans. This effect was attributed to increases in blood oxygenation. In contrast, Schwarzbauer and Heinke showed that in six out of eight awake humans, THC significantly increased the BOLD signal in the visual cortex with no apparent saturation (32). However, the human visual cortex was shown by Kastrup et al. to undergo the highest THC-induced BOLD signal changes when compared to other cortical regions, including sensorimotor, demonstrating the regional dependence of the BOLD signal during THC (49, 50).

The response to hypercapnia may also differ in rats, although no studies of saturation of the BOLD signal in a rat model under conditions similar to the present study have been conducted. Hovarth et al. demonstrated a hypercapnia-induced increase in global brain $CMRO_2$ of over 50% in rats (14), and Berntman et al. demonstrated a 30% increase (36). These results suggest that the non-significant increase in the BOLD signal observed in the present study may be due in part to increased oxygen extraction, and not

to saturation effects (See below for further discussion of this point).

Whether saturation could occur with brain activation is also uncertain. While human studies with graded visual stimulation by Zhu et al. (10) showed no saturation of the BOLD signal, Rees et al. (51) showed the BOLD response in the auditory cortex to high frequencies of word presentation to be non-linear and saturable. The authors postulated that this effect was due to non-linearity in the relationship between BOLD contrast and deoxyhemoglobin concentration, which may have been due to non-linear oxygen extraction when the frequency of word presentation was high. Saturation of BOLD contrast in the present study due to non-linear behavior at high oxygen saturations cannot be ruled out without further studies.

In addition to saturation effects, another factor that could have depressed the BOLD signal, if it had ocurred to any large extent, was hemodilution. Throughout the entire experiment, ten 0.7 cc arterial blood samples were taken, 35% of the rat's total experimentally determined blood volume of 20 cc (52). If this blood were immediately replaced by fluids not containing hemoglobin, then the resulting hemodilution could significantly depress the BOLD signal because of the greater oxygen extraction fraction and hence greater concentration of deoxyhemoglobin (53, 54). However, following significant blood loss it has been shown that decreases in hematocrit lag behing the initial decrease in blood volume by 72 hours (55, 56). Since the time course of this experiment was four hours, hemodilution, and therefore any resulting depression of the BOLD signal, was minimal.

Until recently the degree to which CBF, CBV, and intravascular susceptibility influenced the BOLD signal were not well known. Several studies using exogenous

intravascular contrast agents or high-field magnetic resonance spectroscopy have shown that intravascular susceptibility, and not CBV, is the predominant mechanism of BOLD contrast (39, 40, 41). The principle determinants of intravascular susceptibility are $CMRO_2$, an isolated increase in which causes the BOLD signal to decrease because it results in more local paramagnetic deoxyhemoglobin, and CBF, an isolated increase in which causes the BOLD signal to increase due to a "wash-out" of local deoxyhemoglobin, replacing it with oxyhemoglobin (18, 57). In terms of the discussion above, the findings of the present study could also be viewed as consistent with a significant increase in $CMRO_2$, since there was a significant increase in the FAIR signal, which is dependent on CBF, but no concomitant significant increase in BOLD signal.

The findings of Hyder et al. (5, 21), who demonstrated tight correlation between CBF and tissue oxygen delivery for a wide range of cortical activity, including somatosensory stimulation with α-chloralose anesthesia, may be extended to help explain the findings in the present study. This near-stoichiometric relationship of CBF and $CMRO_2$ is postulated to be a consequence of a changing effective oxygen diffusivity of the capillary bed, linked to alterations in CBF and $CMRO_2$. Several determinants of the relative oxygen diffusivity have been put forward by theorists, such as capillary geometry and number, blood volume and capillary dilation (58, 59), decreases in the number of plasmatic capillaries (60, 61, 62), and intracapillary erythrocyte stacking (63, 64). In this model, during neuronal activation alterations of the above factors and mechanisms are triggered by changes in $CMRO_2$ and CBF to produce the necessary oxygen diffusivity to maintain their near-stoichiometric correlation (5, 21). It is reasonable to assume that some of these factors may be regulated more by CBF than by $CMRO_2$, such as blood

volume, and under the experimental conditions of our study may result in a higher oxygen diffusivity and therefore $CMRO_2$. The present study is the first to examine the CBF response to stimulation after multiple exposures to THC in rat and is not a quantitative study. Further, the oxygen diffusivity under these experimental conditions is unknown and attempting to quantify $CMRO_2$ is beyond the scope of this study.

The post-THC activation data confirm the results of previous studies that demonstrated an increase in somatosensory activation of blood flow following a brief period of hypercapnia (37, 38). In the study by Bock et al. (38) the signal of P-WI doubled while that of T_2^*-weighted images did not significantly increase 25 minutes after a period of brief hypercapnia. These are similar to the results for post-THC Run 1, where a significant 81±63% increase in FAIR signal was demonstrated, with no accompanying significant increase in BOLD signal after one period of THC. The results also showed that the effect occurs early after THC, demonstrated by a significant 78±62% increase in the FAIR signal, while the BOLD signal remained unchanged, in images aquired 6.5-13.5 minutes after the first exposure to THC. In addition the results showed that marked up-regulation of somatosensory activation of the blood flow response was possible with multiple exposures to THC, demonstrated by a 240±54% increase in the FAIR signal after three exposures to THC. The fact that the FAIR signal in each subsequent post-THC run was significantly greater than in the previous run suggests that the mechanism responsible for the up-regulation remained active for at least several hours following the initial exposure to THC, allowing for an additive effect of multiple exposures.

Increased excitability in individual cortical neurons (65, 66) as well as electroencephalographic changes consistent with cortical arousal (67) have been reported

to occur after brief exposure to CO_2. This suggests that in addition to the direct vasodilatory effect of CO_2, which is likely mediated by compounds such as nitric oxide and adenosine, there may be indirect vasodilation triggered by a cortical arousal response (68, 69). This arousal response may result from increased excitation in the cholinergic pathways that connect the hypothalamus and reticular thalamic nuclei to the cerebral cortex (67, 70, 71). In lightly anesthetized animals, CO_2 was shown to induce cortical arousal by increasing excitation in these pathways (68, 72), while lesioning these pathways was shown to abolish the cortical blood flow response to CO_2 (69).

A recent study by Scharwzbauer and Hoehn in awake humans failed to demonstrate an increase in the functional P-WI response to a visual search task after THC (42). The authors cite differences in stimulation paradigm (visual for humans vs. somatosensory in rats), and CO_2 induced up-regulation of the CBF response due to modulatory effects of α-chloralose anesthesia on the cholinergic system as the most likely reasons for the different outcome of their study. One additional point is that the interval between cessation of THC and the beginning (and end) of data collection in the Schwarzbauer and Hoehn study (3-8 minutes post-THC) is earlier than in the other three studies in rats (25 minutes post-THC for Schmitz et al. (37) and Bock et al. (38), and 6.5-13.5 minutes post-THC Run 1, Set 1 in the present study). Due to the beneficial increase in sensitivity that CO_2 induced up-regulation of the CBF response would offer in human fMRI studies, it would be interesting to see the results of a human study where post-THC stimulation images are aquired after slightly longer time intervals.

Building on the studies of Schmitz et al. (37) and Bock et al. (38), this study is the third that demonstrates up-regulation of the CBF response to stimulation after THC in α-

cholralose anesthetized rats. Anesthesia with α-cholralose may indeed be neccesary for this up-regulation to occur, but no comparable stimulation studies have been done to date using other anesthetics following THC. The increasing up-regulation of the flow response after multiple exposures to THC observed in the present study could be due to lasting up-regulation of cholinergic excitatory pathways by the anesthesia, or to some other mechanism (67, 70, 71). Uuntil additional studies are performed in humans, and in rats with different anesthetics, it is prudent to use caution when interpreting the results of any study that significantly alters CBF and/or $CMRO_2$ using any pharmacological or other exogenous means, because of the increasing functional-hemodynamic uncoupling observed in this study.

Conclusion. In this study fMRI evidence of increasing functional-hemodynamic uncoupling has been demonstrated, both in non-activated cortex during hypercapnia, and with somatosensory activation after recovery from multiple episodes of hypercapnia. One explanation that could account for these findings is that the BOLD signal became saturated because of non-linear behavior of BOLD contrast at high oxygen saturations. After considering the most recent theories on the relationship between CBF and $CMRO_2$, and on the determinants of BOLD contrast, the results are also consistent with progressive increases of $CMRO_2$ with stimulation after multiple exposures to hypercapnia. In light of these findings, caution should be used when interpreting fMRI measurements taken subsequent to the administration of any exogenous substance which significantly increases CBF and/or $CMRO_2$.

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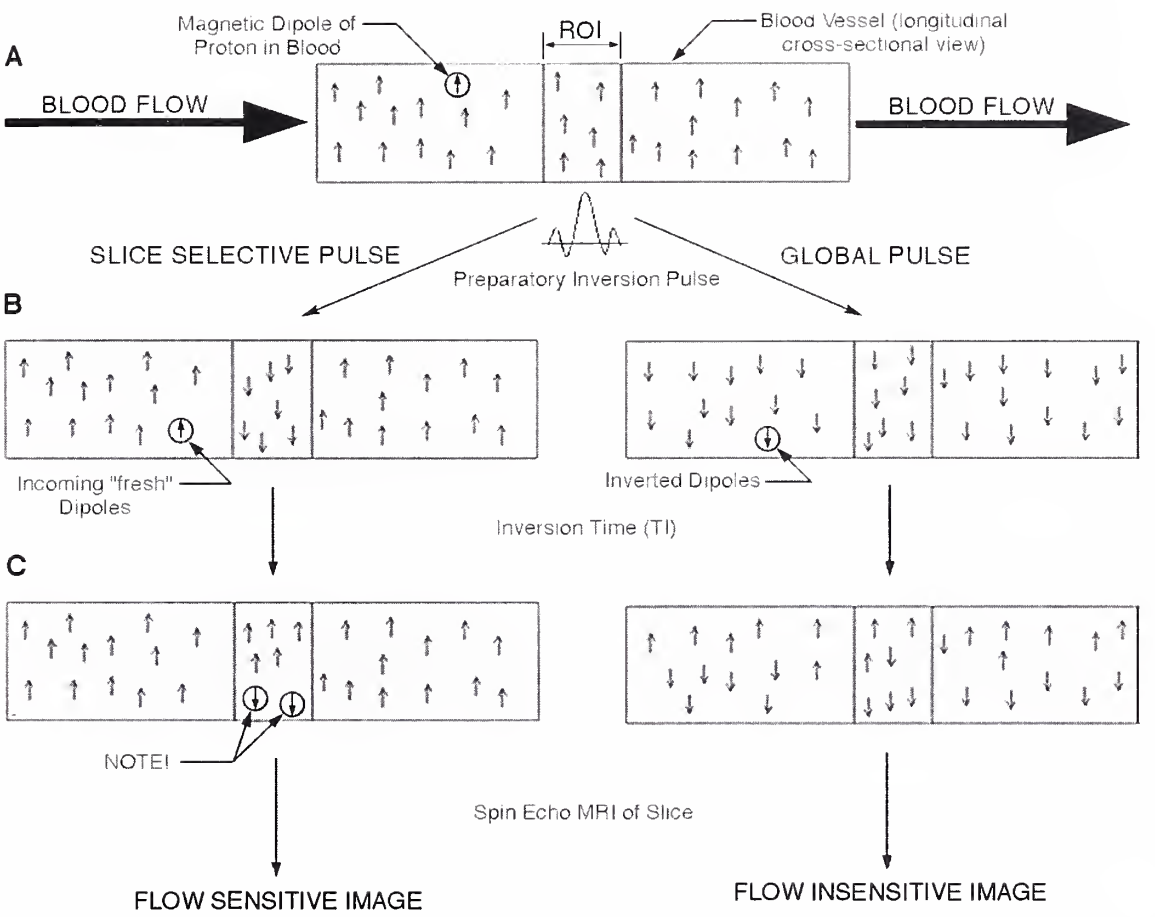
FIG. 1. Depiction of how the Flow-sensitive Alternating Inversion Recovery (FAIR) technique facilitates the acquisition of perfusion-weighted images (PW-I). The five large rectangles above represent longitudinal cross-sections of the same blood vessel at various times throughout the FAIR protocol. Blood flow is from left to right. The region of interest (ROI) is the segment of the vessel from which data are collected that are later used to produce PW-I. **a**) When the subject is in the bore of the magnet, the tiny magnetic dipoles (spins) of all hydrogen (H) nuclei align themselves parallel or antiparallel to the strong constant magnet field. A slightly greater percentage align parallel to the field, a lower energy state, creating a net parallel dipole magnetic moment in any given region of the tissue. **b**) The spins are inverted anti-parallel to the static magnetic field using a preparatory radiofrequency inversion pulse in conjunction with an alternating magnetic field. With the slice-selective pulse, only spins within the ROI are inverted. With a global inversion pulse all spins are inverted. **c**) After the preparatory inversion pulse the spins are given time to "relax," or re-align themselves parallel to the static magnetic field. After a certain inversion time (TI) has passed, a gradient echo EPI image of the ROI is acquired. The slice-selective pulse confers flow sensitivity to the image because uninverted "fresh" spins flow into the ROI causing it to have a faster rate of relaxation than when the ROI is given a global pulse. The faster relaxation rate in the slice selective image results in greater signal intensity in the subsequent EPI image. The flow-insensitive image is subtracted from the flow-sensitive image resulting in a flow-weighted image.

FIGURE 1


A
Magnetic Dipole of Proton in Blood
ROI
Blood Vessel (longitudinal cross-sectional view)
BLOOD FLOW
BLOOD FLOW
SLICE SELECTIVE PULSE
Preparatory Inversion Pulse
GLOBAL PULSE
B
Incoming "fresh" Dipoles
Inverted Dipoles
Inversion Time (TI)
C
NOTE!
Spin Echo MRI of Slice
FLOW SENSITIVE IMAGE
FLOW INSENSITIVE IMAGE

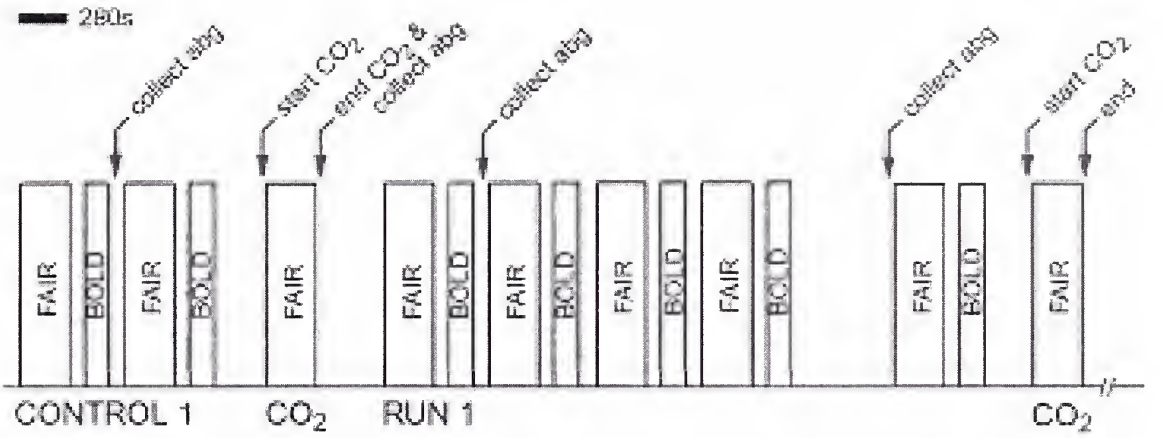


FIG. 2. Depiction of the first run of the experimental protocol. Initially two sets of FAIR and BOLD (CONTROL) images were acquired with collection of an arterial blood sample for blood gas analysis taken in between. This was followed by induction of hypercapnia (CO_2) while a set of FAIR images was acquired, followed by another collection of an arterial blood sample for blood gas analysis. Then there was a five minute wash-out period followed by 4-6 sets of FAIR and BOLD images (Run1). An arterial blood sample was collected 13.5 minutes after cessation of CO_2 administration. There was a 10 minute pause before beginning the second run of the protocol, identical to the first run except only one set of pre-hypercapnia images was collected. The third run of the protocol was performed identical to the second run. See Methods section for details. abg = arterial blood gas.

FIG. 3. Bar graphs depicting representative examples of data acquired from one animal during the entire experimental time course. Data were normalized to the respective pre-hypercapnia controls. **a**) The FAIR signal from the unstimulated cortex increased 60-70% with hypercapnia (signified by THC in the graphs above) and then returned to baseline after each period of THC. With stimulation the FAIR signal progressively increased after each period of THC. **b**) The BOLD signal from the unstimulated cortex also increased with THC and then returned to baseline by the second set of post-THC images in each of the three post-THC runs. The BOLD signal with stimulation increased after the first exposure to THC, then leveled-off after the second and third exposures to THC. UNSTIM = Signal from unstimulated cortex. STIM = Signal from stimulated cortex.

FIGURE 3

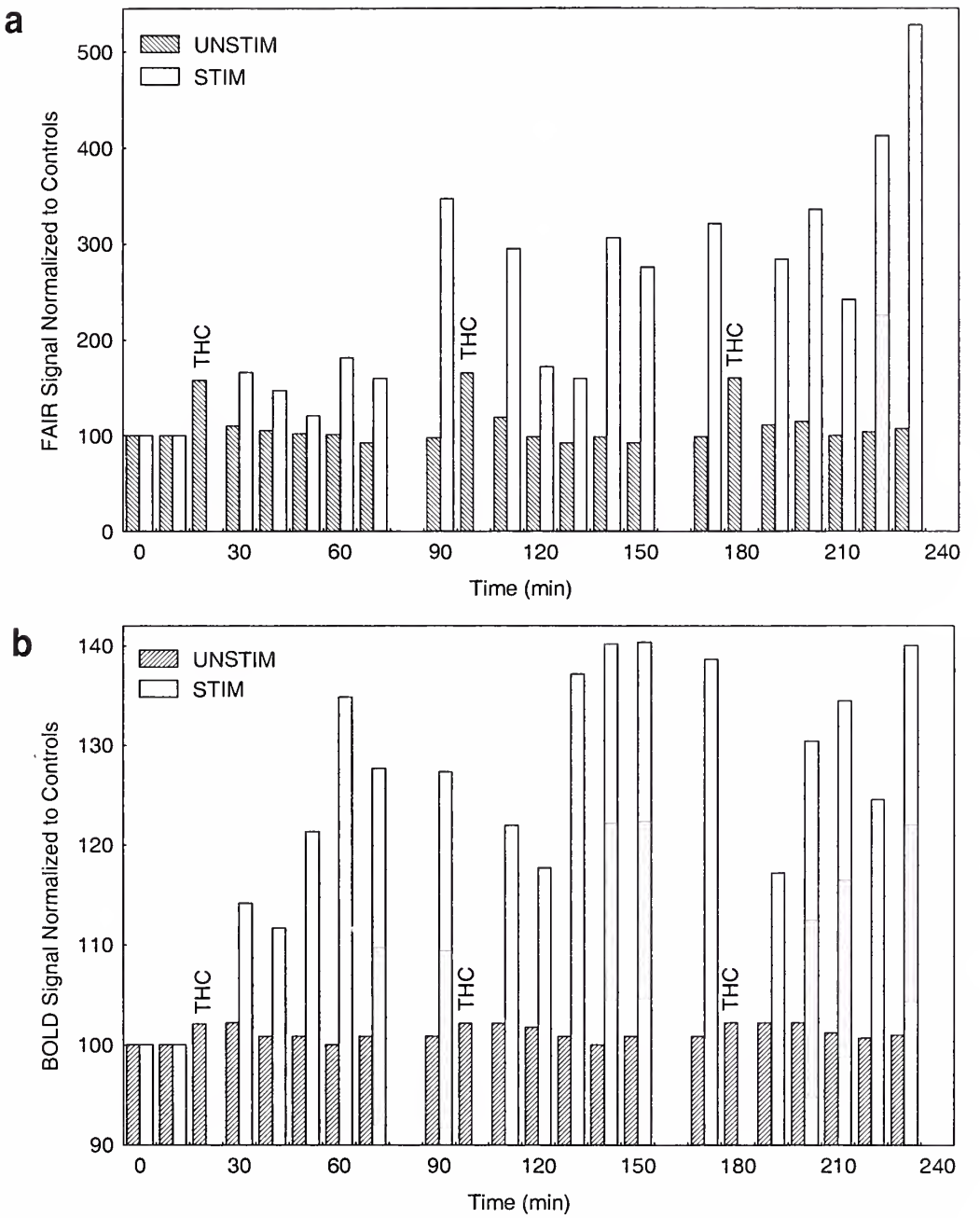
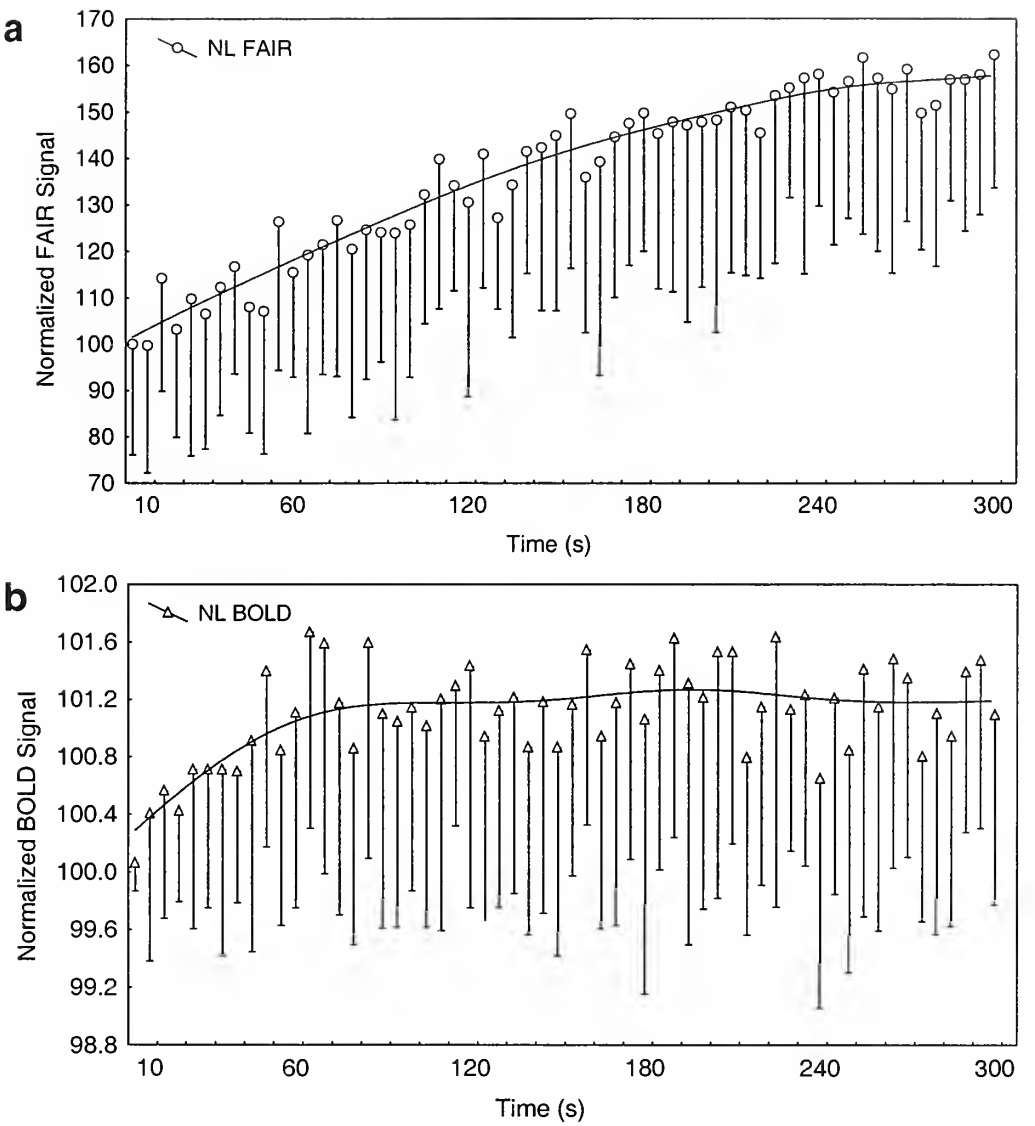

a
UNSTIM
STIM
FAIR Signal Normalized to Controls
500
400
300
200
100
0
THC
THC
THC
0
30
60
90
120
150
180
210
240
Time (min)
b
UNSTIM
STIM
BOLD Signal Normalized to Controls
140
130
120
110
100
90
THC
THC
THC
0
30
60
90
120
150
180
210
240
Time (min)

FIG. 4. Line plots representing the mean grouped FAIR and BOLD signal intensity data from the unstimulated right cortical hemisphere for all three runs during hypercapnia induction. Data were normalized to respective pre-hypercapnia controls. **a**) At image 20 the FAIR signal became significantly elevated vs. pre-THC control and remained significantly elevated until the end of the experiment, with a trend toward greater significance with time as the FAIR signal increased during exposure to THC, with p values ranging from $p = 0.039$ for image 20 to $p = 0.000008$ for image 60. **b**) The BOLD signal reached a plateau at 100 seconds and did not become significantly increased from pre-THC controls at any time throughout the induction period of THC. A regression line was calculated for both data sets using distance-weighted least squares with exponential smoothing. NL FAIR = normalized FAIR signal; NL BOLD = normalized BOLD signal.

FIGURE 4


a
NL FAIR
Normalized FAIR Signal
170
160
150
140
130
120
110
100
90
80
70
10
60
120
180
240
300
Time (s)
b
NL BOLD
Normalized BOLD Signal
102.0
101.6
101.2
100.8
100.4
100.0
99.6
99.2
98.8
10
60
120
180
240
300
Time (s)

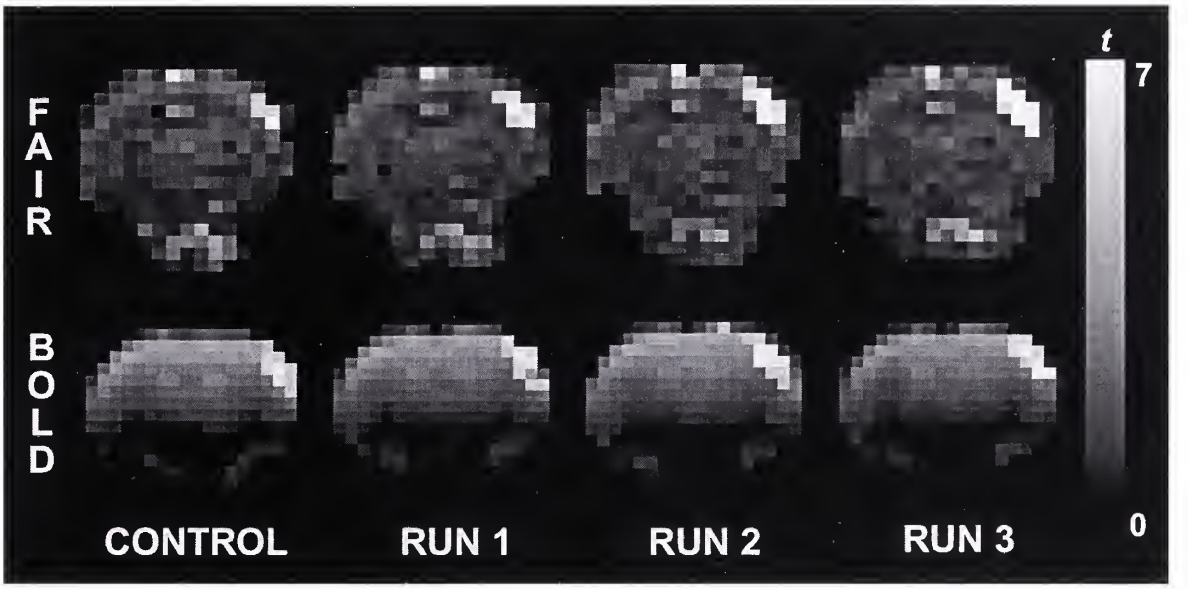


FIG. 5. FAIR and BOLD activation maps obtained during right whisker stimulation at normocapnia, before any CO_2 was administered (Control), and at normocapnia, 35 minutes after CO_2 was administered once (Run 1), twice (Run 2), and three times (Run 3). The maps are overlaid on the respective FAIR and BOLD EPI images aquired between periods of stimulation. The level of activation in the FAIR maps significantly increased after each successive run of THC, as depicted by the marked increase in both the number of and strength of activation of significantly activated pixels. The level of activation in the BOLD maps showed a small, but not significant, increase after one exposure to THC, but did not appreciably increase after Run 2 or Run 3 post-THC. A *p*- value of 0.05 is equivalent to $t = 2.5$.

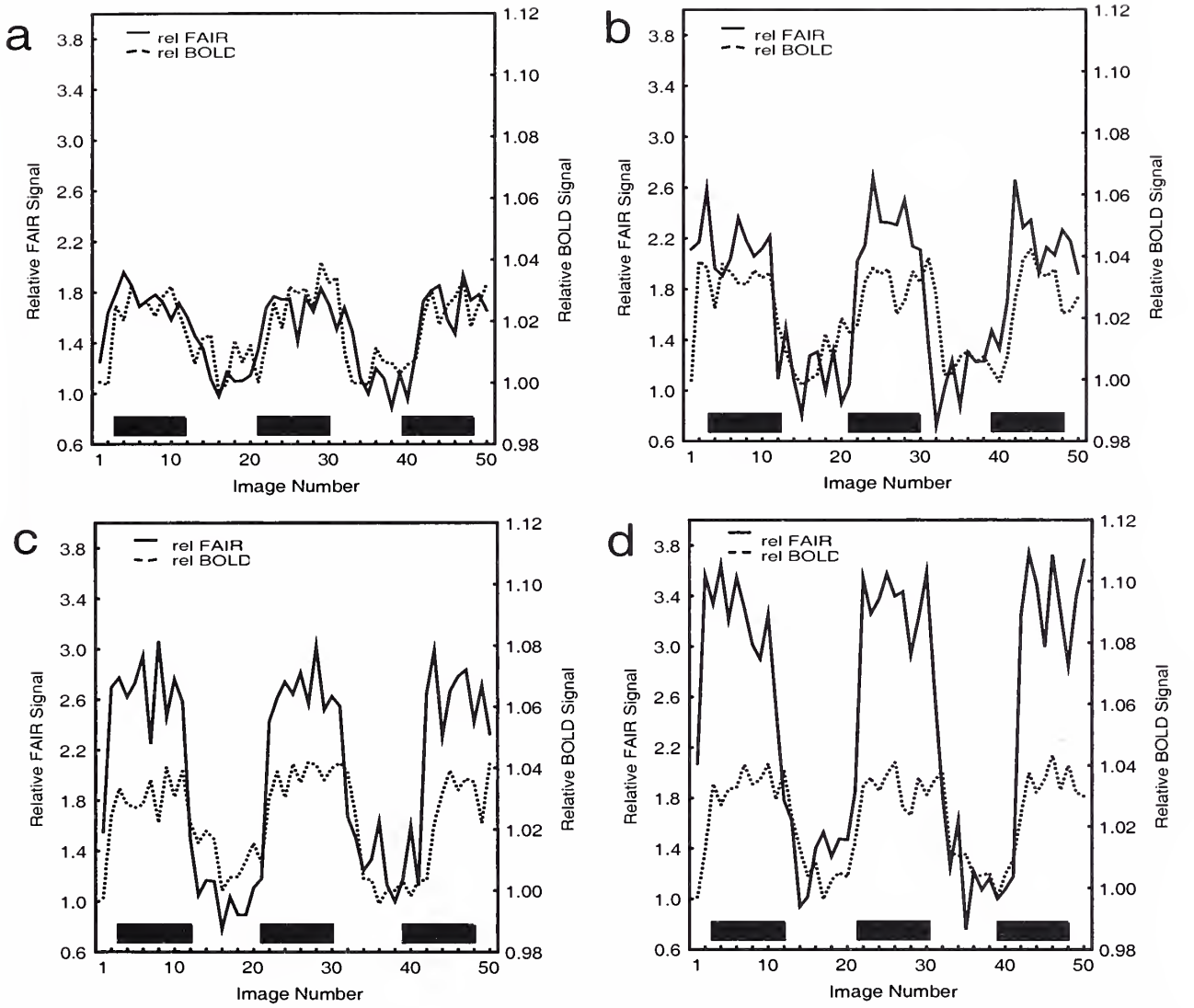


FIG. 6. Time courses of relative changes in the FAIR and BOLD signals in the somatosensory cortex of one rat with whisker stimulation at normocapnia: (**a**) before hypercapnia, (**b**) 35 minutes after one exposure to CO_2, (**c**) 35 minutes after second exposure to CO_2, and (**d**) 35 minutes after third exposure to CO_2. Boxes below time course plots indicate periods of whisker stimulation. rel FAIR = relative change of FAIR signal; rel BOLD = relative change of BOLD signal.

FIG. 7. Bar graph representing mean FAIR and BOLD signal intensity increase in the left somatosensory cortex during right whisker stimulation performed during each of the four periods of normocapnia, which consisted of a pre-THC control, and runs following the first, second, and third exposures to THC. The data were averaged over all animals and grouped as pre-THC control, or post-THC Run 1, 2, or 3. Data were normalized to percent increase of pre-THC controls, which were set to 100%. In the control group stimulation produced a raw signal increase in the whisker barrel of 31.0±5.9% in the FAIR images and 1.84±0.40% in the BOLD images. After one exposure to THC the raw FAIR signal increased by 81±63% and the raw BOLD signal increased by 27±54% over control. After the second exposure to THC the raw FAIR signal increased by 163±55% and the raw BOLD signal increased by 41±67% over control. After the third exposure to THC the raw FAIR signal increased by 240±54% and the raw BOLD signal increased by 35±53% over control. All three FAIR runs increased significantly over control (p = 0.021, p = 0.0002, p = 0.000002), while none of the BOLD runs were significantly increased (p = 0.219, p = 0.098, p = 0.081). In addition FAIR post-THC Run 2 was significantly higher than FAIR post-THC Run1 (p = 0.011), and FAIR post-THC Run 3 was significantly higher than FAIR post-THC Run 2 (p = 0.027). NL FAIR = normalized FAIR signal; NL BOLD = normalized BOLD signal.

FIGURE 7

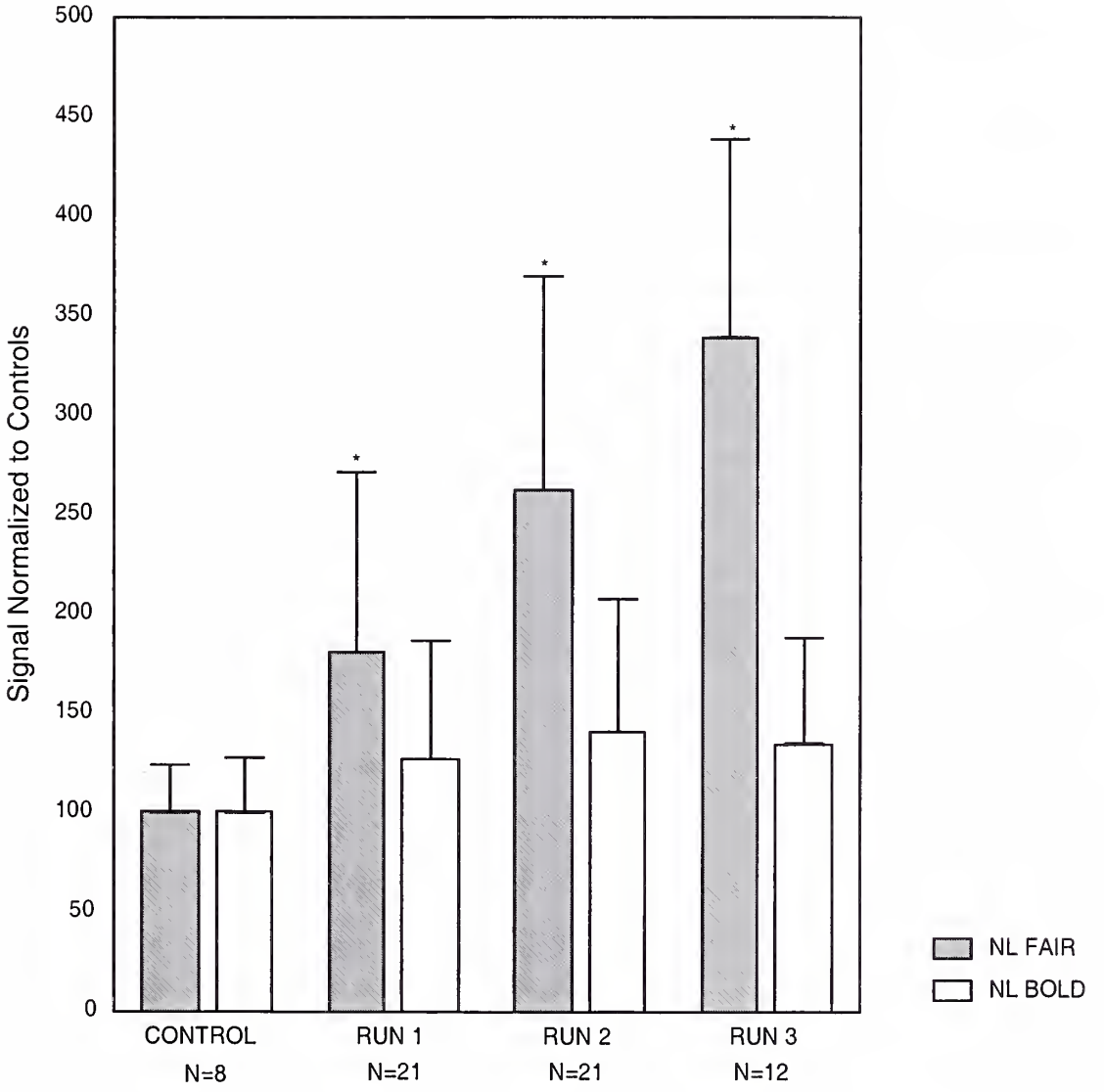

Signal Normalized to Controls
500
450
400
350
300
250
200
150
100
50
0
*
*
*
CONTROL
N=8
RUN 1
N=21
RUN 2
N=21
RUN 3
N=12
NL FAIR
NL BOLD

FIG. 8. Bar graph representing mean FAIR and BOLD signal intensity increase in the left somatosensory cortex during right whisker stimulation performed 6.5-13.5 minutes after cessation of first exposure to THC. Data were normalized to percent increase of pre-THC controls, which were set to 100%. The FAIR signal intensity increased by 78±62% compared to control (p=0.0005). The BOLD signal intensity did not significantly increase from control (-5±18%; p=0.501). NL FAIR = normalized FAIR signal; NL BOLD = normalized BOLD signal.

FIGURE 8

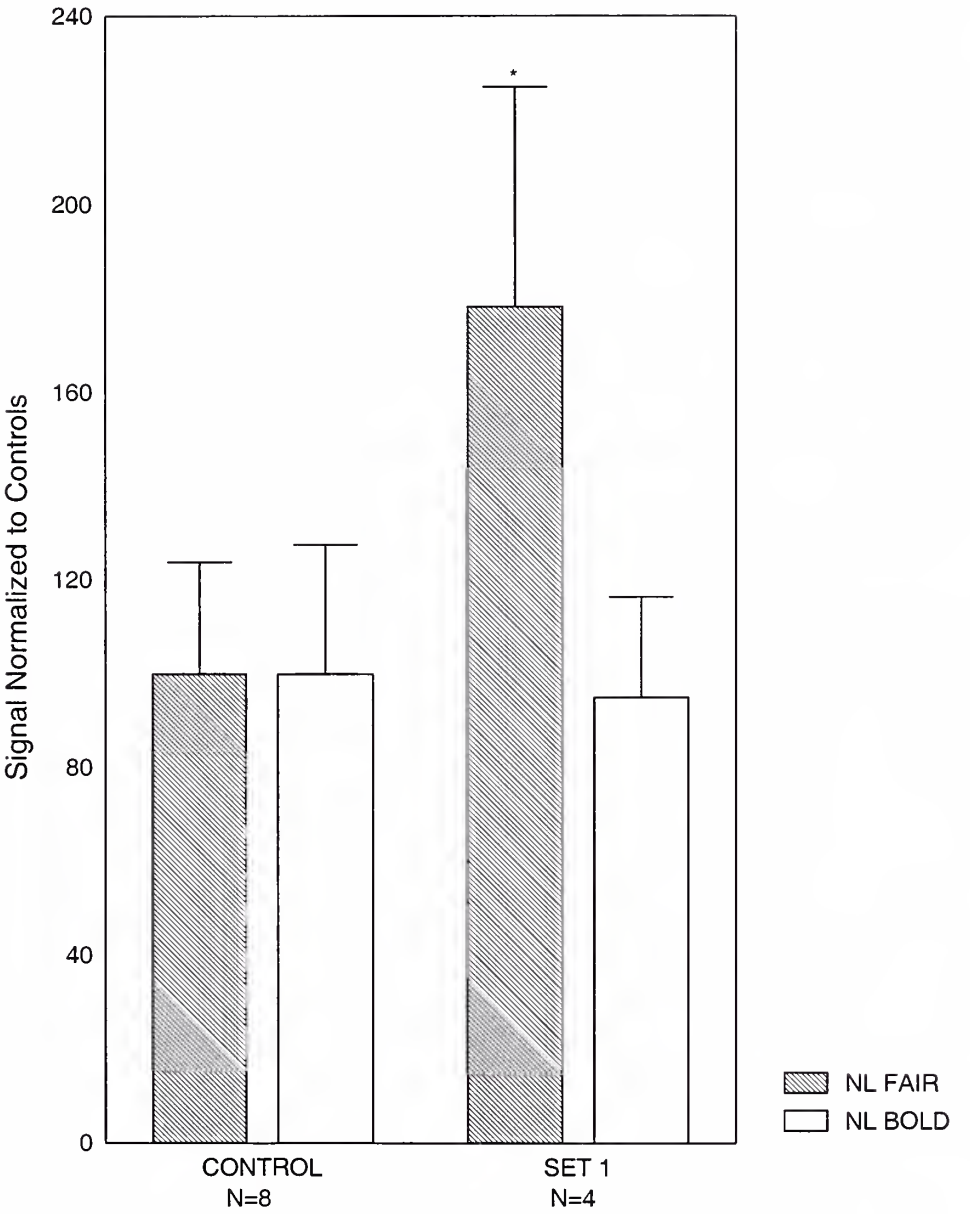

240
200
160
120
80
40
0
Signal Normalized to Controls
*
CONTROL
N=8
SET 1
N=4
NL FAIR
NL BOLD

Table 1. Physiologic Parameters Before, During, and After Three Periods of Transient Hypercapnia Induced by Ventilation with 10% CO_2[a]

	Control	Hypercapnia 1	Run 1	Hypercapnia 2	Run 2	Hypercapnia 3	Run 3
pCO_2 (mmHg)	31.3±7.7	60.8±9.6	28.5±6.0	60.3±9.8	29.0±4.0	59.5±7.0	35.3±8.7
pO_2 (mmHg)	117.3±11.7	192.5±20.4	127.8±17.5	179.8±19.9	121.5±15.6	175.5±19.0	109.0±8.2
pH (units)	7.27±0.08	7.04±0.02	7.28±0.05	7.07±0.05	7.27±0.05	7.07±0.03	7.29±0.02
MABP (mmHg)	103±3	106±3	106±4	102±7	103±9	99±8	103±8
Temp. (°C)	37.2±0.2	37.2±0.2	37.2±0.2	37.2±0.2	37.3±0.2	37.2±0.2	37.2±0.2

[a]Values are expressed as means±standard deviation. MABP, mean arterial blood pressure; Temp., body temperature in degrees Celcius; pCO_2, arterial partial pressure of carbon dioxide; pO_2, arterial partial pressure of oxygen.

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